Studies on Enzyme Action.—Lipase : II.

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The following account has reference to the pursuance of the inquiry into the nature of the process whereby the fats are hydrolysed under the influence of lipase*; the experiments have been made in the hope of discovering an explanation of the selective power which the enzyme undoubtedly displays, as it promotes by preference the hydrolysis of ethereal salts of the higher fatty acids such as are present in the natural fats.

The active material used, as a rule, has been the finely ground air-dried residue which is left on depriving crushed castor oil seed of oil by means of ether; although but slightly active towards ethereal salts other than fats, it is easily prepared of uniform quality and does not soon deteriorate when kept. In all cases, the effect produced in blank control experiments is allowed for. As a rule hydrolysis took place at the temperature of the laboratory.

Operating with what appear to have been highly active materials, Kastle and Loevenhart† were able by means of hepatic and pancreatic lipase to effect the hydrolysis of several ethereal salts derived from the lowest terms of the acetic series of acids. The results they obtained in two series of experiments are as follows, the figures representing the percentage amount of salt hydrolysed after 15 minutes at 40° :—

Ethylic formate	1·60	1·75
„ acetate	0·93	1·75
„ propionate	1·05	2·87
„ butyrate.....	3·13	4·37

As the formate is readily attacked by water alone, they regarded the values for this salt as high; unfortunately they made no control experiments

* Part I, see 'Roy. Soc. Proc.,' B, vol. 76, p. 606.

† *Loc. cit.* We have not yet had the opportunity of consulting Kastle's recent publication.

with any of the salts. They came to the conclusion that the higher the molecular weight of the acid, the more readily is its ethylic salt hydrolysed by lipase.

Ricinus lipase, at least in the form in which we have used it, has a very slight action on ethylic acetate, but gradually hydrolyses the butyrate. Alone it produces little or no effect, mixtures of 2 grammes of seed residue, 3 c.c. ethylic butyrate and 200 c.c. water requiring for neutralisation at the end of 21 hours only 0·7 c.c., after 45 hours 1 c.c. and after 117 hours 1·75 c.c. of normal alkali.

In presence of dilute N/5 acetic acid, action takes place slowly, as shown in the following table—in which the values represent the number of cubic centimetres of normal alkali neutralised:—

	A. 2 grs. seed residue. 20 c.c. acetic acid. 200 c.c. water.	B. 3 c.c. ethylic butyrate. 20 c.c. acetic acid. 200 c.c. water.	C. 2 grs. seed residue. 20 c.c. acetic acid. 200 c.c. water. 3 c.c. ethylic butyrate.
21 hours ...	0·18	0·2	3·2
45 „ ...	0·20	0·2	7·02
69 „ ...	0·20	0·2	10·10
112 „ ...	0·20	0·25	15·3

The extent to which hydrolysis is effected depends both on the amount of enzyme and, up to a certain point, on the proportion of acid present; thus the following results were obtained, using x grammes of seed residue, 0·4 c.c. toluene, 1·5 c.c. ethylic butyrate, y c.c. N/5 acetic acid, water to 100 c.c.

Seed residue.	Cubic centimetres of acetic acid.	Cubic centimetres of alkali required by acid liberated after—		
		19 hours.	43 hours.	67 hours.
gramme.				
1	2	3·5	8·5	13·0
1	5	6·5	11·5	16·0
1	10	7·0	13·0	16·5
1	20	9·6	19·5	
0·5	3	3·0	5·0	7·5

The extent to which the activity of the enzyme is influenced by acids is illustrated in the following table, in which the values represent the number of cubic centimetres of a normal solution of caustic soda required to neutralise the acid liberated in a series of comparable experiments, in each

of which 0·3 gramme of seed residue, 5·0 grammes of castor oil and 5 c.c. acid of the concentration stated at the head of the table were digested together during 20 hours.

Concentration of acid...	N/100.	N/50.	N/17.	N/10.	3N/10.	N/2.
Acetic.....	5·45	14·9	15·4	14·6	14·6	13·6
Succinic	2·8	14·6	15·5	15·4	14·3	12·2
Citric	7·25	15·3	15·2	14·7	7·3	1·1
Tartaric	6·95	15·4	14·5	14·2	2·0	

That the strength of the acid is a factor in the action can scarcely be doubted when the above values are contrasted with the values deduced from the electrical conductivity given by Kohlrausch :—

Acetic acid	K =	0·0018
Succinic „		0·006
Citric „		0·082
Tartaric „		0·097

It is easy to understand that the acids produced when natural fats and oils are hydrolysed have little or no influence on the enzyme, as they are not only weak acids, but also very slightly soluble in water; the influence of the acid therefore is soon at a maximum.

Dr. Nicloux, in drawing attention to his work on Ricinus Lipase,* has stated that the cytoplasm separated from castor oil seed "acts on fat in the same way as an enzyme and follows all the laws of enzyme action"; yet he concludes, that, "Nevertheless the active substance of which cytoplasm is but probably the support is not an enzyme; this substance"—which he proposes to call lipaseidine—"is destroyed by water as soon as it is no longer protected by fats."

We cannot help thinking that this conclusion is illogical and that the destruction of the lipase prepared by Nicloux is probably to be traced to an admixed proteoclastic enzyme rather than to water. The substance in question was obtained by expressing the oil from the seed, then centrifugalising the oil to separate the suspended solid matter and washing this latter free from oil with carbon bisulphide. It was intensely active as a lipoclast.

The material we have used, though free from oil, is but slightly affected by water or dilute acids. After such treatment, however, it loses to a considerable extent the property of causing oil to form an emulsion with water;

* *Ibid.*, B, 1906, vol. 77, p. 454.

we are inclined to attribute its inferior activity principally to this effect. Our results are as follows:—

0·3 gramme of seed residue was digested with 3 c.c. of water during the time stated, 5 grammes of castor oil and 3 c.c. N/5 acetic acid were then added; the mixture, having been well shaken, was then maintained during 21 hours at 25°. The figures represent the number of cubic centimetres of N/NaOH required to neutralise the liberated acid.

Time	0 mins.	13·6
	10 „	12·1
	30 „	12·4
	60 „	10·6
	120 „	10·9

0·3 gramme of seed residue was digested with 3 c.c. N/5 acetic acid during the time stated, 5 grammes seed residue and 3 c.c. water were added, the mixture well shaken and then maintained during 19 hours at 25°; the number of cubic centimetres of N/NaOH required to neutralise the acid liberated at the end of this period was as follows:—

Time	0 mins.	15·8
	10 „	14·5
	30 „	15·3
	60 „	10·9
	120 „	11·8
	180 „	10·9
	94 hours	12·4

A sample was allowed to remain in contact with the acetic acid during 168 hours and then shaken with it in the shaking machine during 3 hours; after 19 hours 14·65 c.c. N/NaOH was required to neutralise the acid which was liberated from 5 grammes of castor oil.

Several experiments were made in which a large excess of water was taken and the mixture shaken in the shaking machine in order to reduce to a minimum the decrease in the amount of fat hydrolysed due to the differences in the character of the emulsion. 0·3 gramme of seed residue and 0·4 c.c. of toluene was allowed to remain in contact with (*a*) 10 c.c. N/5 acetic acid and 10 c.c. water, (*b*) 20 c.c. water, during the time stated; 5 grammes castor oil was then added and to (*a*) 10 c.c. water and to (*b*) 10 c.c. N/5 acetic acid, and the mixtures were shaken during 5 hours at 15° to 17°. The number of cubic centimetres of N/NaOH required to neutralise the acid liberated was

(*a*)		(*b*)	
Time 90 hours	3·2	Time 0	5·6
3 weeks	2·8	90 hours	2·6
		3 weeks	1·7

According to Nicloux, glycerol retards the hydrolysis. Our experiments indicate that up to 25 per cent. glycerol has little, if any, influence; in larger proportion, it certainly retards hydrolysis. All observers agree that alcohol hinders the change: our experiments show that its influence is approximately proportional to the amount which is present.

The ethereal salts which are hydrolysed under the influence of lipase are all compounds of the type

$$R'.CO.OX'.$$

Since R′ and X′ may be varied within wide limits, it cannot well be supposed that the selective action of the enzyme is exercised with reference either to R′ or X′: consequently the controlling influence must be attributed to the carboxyl radicle (CO.O); the enzyme must be so constituted that it can "fit itself to this group."

The problem to be solved is—why should ethereal salts derived from the lower terms of the acetic series be so much less readily hydrolysed than the higher? The differences in stability do not account for the differences in behaviour of homologous salts; in fact, ordinary hydrolytic agents appear to act more readily on the lower terms. Nor can the difference be attributed to the destruction of the enzyme by the acid which is liberated from the salt, as this destructive effect can be avoided by diluting the solutions to the necessary extent.

Our experiments have led us to form the provisional hypothesis that the hydrolysis of the ethereal salt by lipase involves the direct association of the enzyme *with the carboxyl centre* and that such association may be prevented by the "hydration" of this centre: consequently, that those salts which are the more attractive of water will be the less readily hydrolysed. The facts generally seem to be in accordance with this view, inasmuch as the solubility in water of ethereal salts diminishes as the series is ascended; salts such as ethylic formate and acetate undoubtedly tend to form hydrates (hydrols) in solution, such as

$$CH_3.CO.OEt + OH_2 = CH_3.C(OEt)<^{OH}_{OH}$$

The following comparative results are of interest from this point of view:—

0·3 gramme of seed residue.
1 c.c. ethereal salt.
0·1 c.c. of toluene.
5 c.c. of N/5 acetic acid.
Water to 50 c.c.

Cubic centimetres N/5 NaOH required to neutralise Acid liberated by Lipase after—

	29 hrs.	6 days.
Ethylic malonate	5·5	10·35
„ succinate	11·75	30·75
„ malate	3·5	13·5
„ tartrate.....	0·5	0·5

The formulæ of the four salts are as follows:—

$CH_2\begin{matrix}COOEt\\COOEt\end{matrix}$	$\begin{matrix}CH_2.COOEt\\CH_2.COOEt\end{matrix}$	$\begin{matrix}CH_2.COOEt\\CH(OH).COOEt\end{matrix}$	$\begin{matrix}CH(OH).COOEt\\CH(OH).COOEt\end{matrix}$
Et. malonate	Et. succinate	Et. malate	Et. tartrate

In view of the position which it occupies between the succinate and tartrate and of its relation to ethylic malonate, the behaviour of ethylic malate is of special interest.

Other noteworthy results are the following:—

	20 hrs.	44 hrs.
Ethylic acetate	0·75	0·7
Ethylic butyrate	6·0	10·0
Amylic acetate	2·5	4·5
Ethylic malonate	5·5	8·0
Ethylic dimethylmalonate	0·75	0·75
Ethylic benzoate	0·75	1·0
Methylic salicylate	0·5	0·5
Ethylic mandelate.....	0·5	0·5
Methylic oxalate	—	—

The fact that amylic acetate, which is far less soluble in water than ethylic acetate, is more susceptible to lipase is an interesting confirmation of the explanation given above.

As it appeared possible that the differences in the rates at which different salts were hydrolysed might be due to the poisoning of the enzyme, the finely ground seed residue was mixed with the ethereal salt and the mixture shaken during an hour; the solid was then filtered off, washed with ether

and dried. This material was used in the following experiments in which 0·3 gramme seed residue, 0·1 c.c. toluene, 5 grammes castor oil, 3 c.c. N/5 acetic acid and 3 c.c. water were digested together. The number of cubic centimetres of N/NaOH required to neutralise the acid liberated during 20 hours was—

Treatment of seed residue.	
Untreated	14·6
Alcohol	9·0
Ethylic butyrate	14·9
Ethylic acetate	14·65
Ethylic tartrate	11·3
Methylic sulphate	0·6

Comparison of Animal with Vegetable Lipase.—Dr. Harden has been good enough to prepare for us at the Lister Institute a quantity of animal lipase, following the directions given by Dakin.* A fresh pig's liver was minced and mixed with Kieselguhr and sand; the expressed fluid (450 c.c.)—a dark red liquid—was then centrifugalised to remove a small amount of suspended jelly. The fluid was diluted with water in various proportions for the experiments. This lipase acted readily on ethylic butyrate and to a very slight extent on ethylic tartrate. The following results were obtained, using 1 c.c. ethereal salt, 50 c.c. lipase solution and 0·2 c.c. toluene:—

Percentage of lipase solution.	Cubic centimetres of N/5 alkali corresponding to acid liberated by enzyme after 48 hours at 25°.	Percentage hydrolysed.
Ethylic butyrate—		
5	35·2	90
$2\frac{1}{2}$	26·95	69
1	20·9	53
$\frac{1}{2}$	17·45	44
$2\frac{1}{2}$ + 10 N/5 acetic acid	20·05	51
Ethylic tartrate—		gramme.
5	2·5	= 0·0515
5 + 10 N/5 acetic acid	1·75	= 0·036

The action of the two lipases was contrasted in a series of experiments using mixtures (*a*) of 1 c.c. of ethereal salt, 0·2 c.c. toluene and 50 c.c. of 2-per-cent. lipase solution, (*b*) of 1 c.c. ethereal salt, 5 c.c. N/5 acetic acid, 0·2 c.c. toluene, 0·5 gramme castor oil seed residue, water to 50 c.c.

* 'Journal of Physiology,' vol. 32, p. 202.

The results show that the difference is probably only one of degree and also illustrate the comparative instability of the animal product.

	Cubic centimetres of N/5 alkali corresponding to acid liberated by enzyme at 25° after—			
	Animal.		Vegetable.	
	20 hours.	68 hours.	20 hours.	68 hours.
Ethylic malonate	9·65	10·35	3·5	7·5
Ethylic dimethylmalonate	4·0	4·35	none	none
Ethylic succinate	22·15	24·9	15·5	25·8
Ethylic malate	7·65	7·85	2·25	6·0
Ethylic tartrate	none	0·3	none	none

From the following results it seems that if only sufficient enzyme be used all ethereal salts are more or less attacked:—

Animal lipase.

1 c.c. salt + 0·2 c.c. toluene + 20 c.c. 2 per cent. liver juice.

Vegetable lipase.

1 c.c. salt + 20 c.c. water + 2 c.c. N/5 acetic acid + 0·2 c.c. toluene + 1·0 gramme seed residue.

	Cubic centimetres of N/5 alkali corresponding to acid liberated by enzyme at 25° after—			
	Animal.		Vegetable.	
	21 hours.	68 hours.	21 hours.	68 hours.
Ethylic mandelate.....	1·4	1·4	1·9	2·0
Ethylic benzoate	2·8	3·9	0·68	0·8
Methylic salicylate	0·3	0·3	0·2	0·4
Ethylic acetate	3·6	10·35	3·3	9·9
Ethylic tartrate.....	0·2	—	1·0	

It is proposed to study the action of lipase very thoroughly from the point of view of the working hypothesis now brought forward. A comparative examination of the enzyme derived from various sources will also be undertaken, both in order to ascertain whether only one form of lipase exists and to obtain the hydrolyst in a really suitable form for the quantitative study of its effects. Should the explanation we have advanced be justified, ethereal salts will be a material at least as valuable as that afforded by the carbohydrates for the comparative study of enzymes and acids as hydrolytic agents.

APPENDIX. (August 14, 1906.)

Since the foregoing account was written, a large number of experiments have been made with the object of contrasting animal lipase with vegetable lipase. We have been led gradually to recognise that in the case of the former especially, if an effective comparison is to be made between ethereal salts, it is an essential condition of success that the substances compared be in solution. We were led to this conclusion in the first place by observing in certain experiments, in which the materials were only partially dissolved, that ethylic propionate was more acted upon than was either the acetate or butyrate; but when solutions of equivalent quantities of the three salts were acted upon by liver lipase, the acetate proved to be the most and the butyrate the least stable. We need scarcely point out that this circumstance renders a strict comparison of ethereal salts which are only very slightly soluble in water a difficult matter; moreover, it must be taken into consideration in connection with our earlier experiments. Probably it is on this account also that the liquid expressed both from the liver and the pancreas acts so slowly and to so slight an extent on natural fats and oils. With these agents, however, it appears to be impossible to secure anything approaching to the complete emulsification of fats which is readily effected by the residue of castor oil seeds; indeed, at present we are inclined to attribute the extraordinary activity of the seed residue to its emulsifying power rather than to any inherent superiority of the enzyme as a lipoclast; but it should be mentioned that any treatment which renders the enzyme in the seed residue inactive also destroys the emulsifying power of the material.* All attempts which we have made to overcome the difficulty referred to by dissolving the fat or oil in neutral liquids such as toluene or ether and then violently agitating the solution with liver juice have proved unsuccessful—such treatment having served only to destroy the enzyme.

In confirmation of the statement already made with reference to the remarkable and, as we believe, significant difference in behaviour of the allied salts, ethylic succinate, malate and tartrate, the following results may be quoted which were obtained by digesting solutions of equivalent quantities of the three compounds with liver lipase. The figures represent the quantity of alkali required to neutralise the liberated acid; at the end of the experiment the succinate was practically all hydrolysed:—

* It is not improbable that the increased activity of pancreatic juice (from a Pawlow fistula) in presence of bile salts, to which Magnus has recently called attention ('Zeits. Physiol. Chem.,' 1906, vol. 48, p. 376), is due to the promotion of emulsification by the salt.

	1 hour.	3 hours.	24 hours.
Ethylic tartrate	0·9	1·2	1·8
„ malate	2·70	5·7	15·0
„ succinate	13·50	14·5	20·4

We have to thank Mr. R. R. Armstrong, B.A., for valuable assistance rendered in the latter part of the inquiry.

The Action of Plants on a Photographic Plate in the Dark.

By W. J. Russell, Ph.D., F.R.S.

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[Plates 19–21.]

It has been shown in former papers that wood has the property of acting in the dark on a photographic plate, when placed in contact or in proximity to it. Not only does wood act in this way, but leaves, seeds, roots, bulbs, and, in fact, with only few exceptions, all vegetable substances act in a similar way. The more important bodies which are without this property are starch, cellulose, gum, sugar, pith, and pollen. To obtain this action on a plate it is necessary that the body used be tolerably dry, or else the moisture contained in it will act on the gelatine of the photographic plate and destroy the picture. The time necessary for the exposure to the plate varies from a few minutes to 18 hours or more. To quicken the action, heat may be applied, but the temperature must not be above 55° C., nor the time of exposure, under ordinary circumstances, longer than 18 hours, or the photographic film will be injured. Any ordinary rapid photographic plate may be used, and its development is exactly the same as that of an ordinary picture. The best and most general method of drying vegetable substances before exposing them to the photographic plate is to place them between pure white blotting paper and subject them to considerable pressure, say from 1 to 5 or 6 tons per square inch. This process has also the advantage of giving a second picture, for it is found that the liquid which has been expressed and absorbed by the blotting paper is capable of acting on a photographic plate, and that it gives a good representation of the plant from which it came (Plate 19, fig. 1, an oak leaf).

Since different woods are capable of acting on a photographic plate it was to be expected that leaves, stems, flowers would do the same. This has